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PRINCIPAL INVESTIGATOR: R. Jeremy Nichols, Ph.D.

CONTRACTING ORGANIZATION: Parkinson's Institute

Sunnyvale, CA 94085

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R. Jeremy Nichols, Ph.D.

E-Mail: jnichols@thepi.org

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14. ABSTRACT
The gene encoding the protein kinase LRRK2 is the most frequently mutated gene in familial Parkinson's disease (PD). The microtubule associated protein Tau is a pathological component of Alzheimer's disease and genome wide associations studies implicate Tau in PD. In several mouse models it has been shown that Tau exhibits a LRRK2 dependent change in phosphorylation status. The confluence of evidence that these two disease proteins may intersect in disease pathogenesis yielded the hypothesis that LRRK2 kinase activity potentiated the phosphorylation of Tau. We originally proposed to investigate known tau kinases as being stimulated by LRRK2 activity to modify tau, but we subsequently identified that LRRK2 can directly phosphorylate Tau. We found that LRRK2 can phosphorylate Tau and that this modification is enhanced in shorter splice forms of Tau (non-441 splice variants) and that addition of tubulin to kinase reactions enhanced the phosphorylation. We mapped 6 LRRK2 phosphosites on Tau using mass spectrometry (T149, T169, T205, S210, T217AND T263). We prepared 15 different Tau phosphomutants and purified them as recombinant proteins from bacteria. We tested the effects of these mutations on tubulin assembly and LRRK2 mediated phosphorylation. We found that only three mutants increased tubulin assembly (Thr169, Thr181 and S210). Unfortunately, we failed to completely ablate LRRK2 phosphorylation of any of the mutants we generated, indicating still more sites of LRRK2 phosphorylation, meaning more studies are required to understand the role of LRRK2 in phosphorylating and regulating Tau function.

15. SUBJECT TERMS
LRRK2, tau, tau splice forms, tubulin, tubulin assembly, phosphorylation, substrate

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Introduction

LRRK2 mutation has been shown to be a causative factor for inherited PD [1-4] and in multiple Genome Wide Association Studies (GWAS) it has also been found to be associated with PD [5-8]. Additionally, mutation in the microtubule associated protein tau locus (MAPT) show clear associations with PD, and has now been classified as a risk factor for PD [7, 8]. Tau protein is well known for its role in Alzheimer's disease, when in the hyperphosphorylated state, tau protein forms paired helical filaments (PHFs) and are pathologically characterized as tangles in post mortem tissue [9-12]. Tau mutations have also been associated with other parkinsonism related disorders such as frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP), Pick's disease, Niemann-Pick disease type C, post encephalitic parkinsonism, as well as geographically characterized taupathies such as amyotrophic lateral sclerosis/Parkinson-dementia complex of Guam and atypical parkinsonism observed in the French West Indies, all reviewed in Ludolph et al. 2009 [11]. These genetic data indicated that there could be a potential role for tau in Parkinson's disease.

Further supporting a role for tau in parkinsonism is the important observation that this is the observation that tau phosphorylation states are altered in mouse models of Parkinson's disease based on LRRK2 mutant expression. In four reported models of mouse LRRK2 mutants, changes in tau phosphorylation at the 202/205 and 396/404 sites were observed [13-16]. The data indicated that more LRRK2 (gene dosage or increased activity) resulted in more phosphorylated tau; conversely, decreases in LRRK2 result in less phosphorylated tau. Since hyperphosphorylated tau contributes to a cardinal feature of Alzheimer's disease, neurofibrillary tangles, there might be pathological connections to Parkinson's disease where NFTs are sometimes observed in post-mortem tissue [Parkinson's Institute Brain Bank].

We originally hypothesized that LRRK2 could potentiate tau phosphorylation via regulation of known tau upstream kinases and set out to investigate this theory. However, during the course of our studies we determined that LRRK2 could phosphorylate Tau directly and with greater efficiency if reactions are performed in the presence of tubulin. The most common familial PD mutation in LRRK2 is known to increase the kinase activity of the enzyme, which could induce tau hyperphosphorylation. We therefore sought to map the sites of LRRK2 phosphorylation on Tau and determine their contribution to tubulin assembly in vitro, a known function of Tau. Our new platform of investigation is to ask: *"What the specific LRRK2 phosphorylation sites on tau are and do these affect tau initiated microtubule assembly?"*

Body

The main goal of our funded work in W81XWH-11-1-**0338** was to answer the question of "*How does LRRK2 kinase activity potentiate Tau phosphorylation*?" We had originally proposed to investigate the potential for LRRK2 regulation of the kinase(s) that phosphorylates Tau. However, during the course of our work, we observed that Tau is directly modified by LRRK2. During the period of our initial observations of this phenomenon, a group published that indeed, LRRK2 can modify Tau splice-form 441 preferentially in the presence of tubulin [17]. Our results presented herein represent our current progress in characterizing the direct modification of Tau by LRRK2.

LRRK2 directly phosphorylates Tau. We first tested the ability of LRRK2 to directly modify Tau in vitro. Tau is found in multiple splice forms and are designated by their amino acid length in amino acids: 441, 412, 410, 381, 383 and 352 [9]. We examined the ability of LRRK2 to

phosphorylate all isoforms using recombinant tau spliceforms and observed that indeed, LRRK2 phosphorylates Tau (**FIGURE 1**). The level of modification of tau on the largets spliceform 441 and the shortest spliceform is low compared to the modification of 412, 410, 381 and 352. These data were intriguing and indicated a difference between substrate selection of LRRK2 for tau and explains previous negative data obtained when examining the modification of Tau441 alone (Nichols and Alessi unpublished data). We next wanted to test if inclusion of tubulin in kinase reactions would alter tau modification because 1) tau binds microtubules and promotes assembly into filaments and 2) LRRK2 has been reported to phosphorylate tubulin. We used the Tau441 and asked if tubulin would affect the amount of modification of tau we observed (**FIGURE 1B).** We found that indeed, LRRK2 modified tubulin and again we observed little modification of Tau441. However, when tubulin is included in kinase reactions, we found that there was a large stimulation of Tau441 phosphorylation **(FIGURE 1B)**. We conclude from these data that LRRK2 can directly phosphorylate tau and that this modification is enhanced by tubulin but not necessary for multiple tau spliceforms.

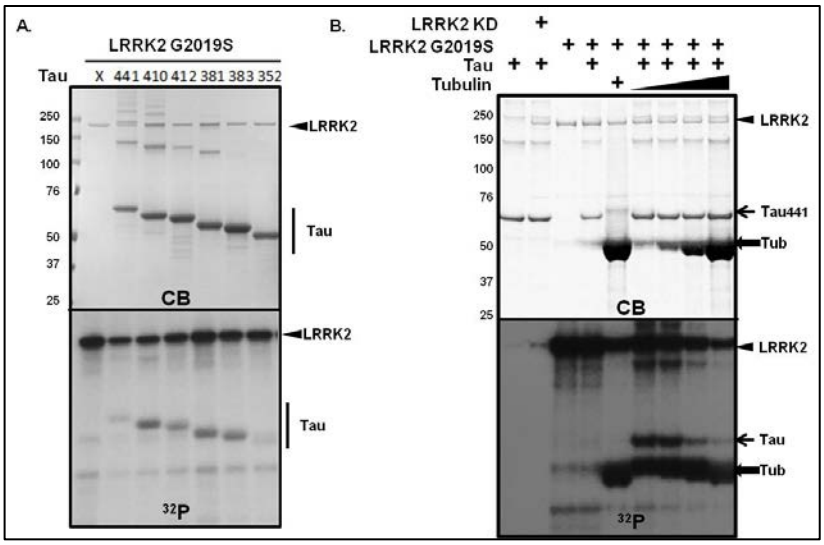

Figure 1. LRRK2 phosphorylates Tau. A) LRRK2 modifies multiple splice variants of Tau. Recombinant protein representing the indicated splice variants of Tau were subjected to in vitro kinase reaction with active LRRK2 G2019S (970-End) protein in with γ32P-ATP. Reaction products were resolved by SDS-PAGE followed by staining with colloidal blue (CB) and phosphorylation was detected by autoradiography (32P). B) The longest isoforms of Tau, 441, was subjected to in vitro kinase reactions in the absence or presence of increasing amounts of porcine tubulin protein with LRRK2 kinase inactive (KD) and G2019S variants. Reaction products were

LRRK2 phosphorylates several sites on Tau. Because we found LRRK2 modified Tau, we next asked what are the *in vitro* LRRK2 phosphorylation sites of Tau? We performed in vitro kinase assays using recombinant LRRK2, tau and tubulin. Using mass spectrometry, we mapped six novel LRRK2 phosphorylation sites on tau, Thr149, Thr169, Thr205, Ser210 and Ser263; these are shown in **FIGURE 2A**. Gratifyingly, one of these sites (Thr205) has been identified in the murine studies we quoted in the original proposal [13-16], revealing a potential physiological link. We did not observe the modification at Thr181 as was reported in Kawakami et al. 2012 [17]. Thr205 phosphorylation is intriguing, as the AT8 antibody referenced in the narrative of the original proposal [13-16] recognizes Tau phosphorylated 205. Our results reveal a novel and more comprehensive modification profile for Tau by LRRK2 that appropriately require follow-up studies. It is important to next validate the sites of LRRK2 phosphorylation on Tau that we have identified and ascribe a functional significance to these modifications.

In order to validate these sites of modification, we generated cDNA mutants encoding the Tau441 variants listed in **FIGURE 2B**. With these mutants we successfully expressed and purified from bacteria, recombinant Tau proteins of these mutants and a representative SDS-PAG of these proteins is shown in **FIGURE 2C**.

A.

Site	Sequence	Mascot Ion score	Modifications	Observed	Actual Mass	Charge	Delta PPM	Start	Stop
T149	(S)KDGTGSDDKKAKGADGKtKIATPR(G)	31.68	Phospho (+80)	632.0725	2,524.26	4	-1.824	132	155
T149	(K)GADGKtKIATPR(G)	48.9	Phospho (+80)	647.8285	1,293.64	2	-1.602	144	155
T169	(R)GAAPPGQKGQANAtRIPAK(T)	19.18	Phospho (+80)	638.3291	1,911.97	3	-1.479	156	174
T169	(K)GQANAtRIPAK(T)	30.99	Phospho (+80)	603.8027	1,205.59	2	-0.8233	164	174
T169	(K)GQANAtRIPAKTPPAPK(T)	21.7	Phospho (+80)	599.9833	1,796.93	3	-1.162	164	180
T205	(R)SGYSSPGSPGtPGSR(S)	49.48	Phospho (+80)	737.3029	1,472.59	2	-1.434	195	209
T217	(R)SRTPSLPtPPTREPK(K)	18.82	Phospho (+80)	581.9641	1,742.87	3	-0.9057	210	224
S210	(R)sRTPSLPTPPTREPK(K)	24.06	Phospho (+80)	581.9636	1,742.87	3	-1.645	210	224
T263	(K)SKIGStENLKHQPGGGK(V)	32.85	Phospho (+80)	606.6343	1,816.88	3	-1.424	258	274

B.

	Name
1	Tau WT
2	T149A
3	T169A
4	T169E
5	T181A
6	S202A
7	T205A
8	S210A
9	T217A
10	T263A
11	S396A
12	S404A
13	202/205
14	396/404
15	202/205/181
16	Tau MUT8

C.

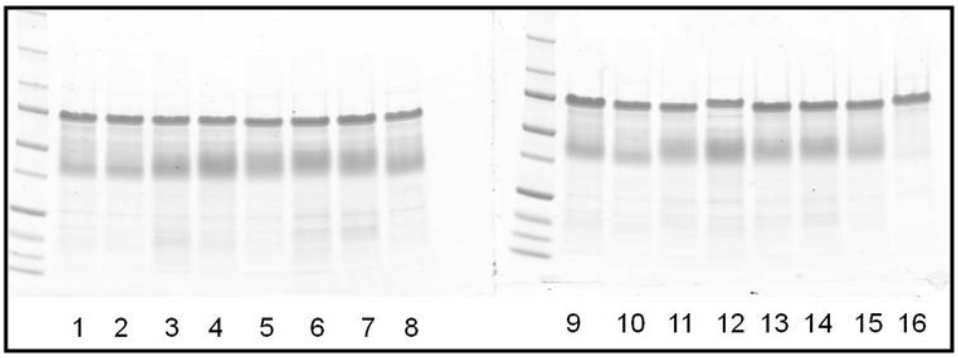

Figure 2. LRRK2 phosphorylation sites on Tau and generation of recombinant protein. A) Recombinant Tau and Tubulin were subjected to in vitro kinase assays with LRRK2 and reaction products were resolved by SDS-PAGE. Bands corresponding to Tau were processed for mass spectrometry using trypsin, chymotrypsin and AspN proteases. Peptides identified, with mascot scores and mass spectrometry data are provided. **B)** List of Tau mutants generated and used in this study. MUT8=all 8 phosphorylation sites mutated in the same construct, except for the 396/404 sites. **C)** Coomassie blue stained SDS-PAG of recombinant His-tagged Tau proteins purified from bacteria resolved on S DS-PAG. Numbering corresponds to **(B).**

Phosphosite mutant effects on Tau mediated tubulin assembly. Tubulin exists in two monomeric forms (α and β) which assemble to form filaments. This assembly can be modeled in vitro using purified tubulin and additional co-factors that promote assembly. These co-factors can be molecular crowding agents such as high glycerol concentrations or specific proteins that enhance the formation of tubules such as MAP2 (microtubule associated protein 2) or tau protein. Indeed, our recombinant Tau does promote tubulin assembly, indicating the physiological competence of our preparations. We examined the effects of the addition of WT or mutant Tau (from **FIGURE 2B**) to tubulin assembly reactions **FIGURE 3**. Here, we used a fluorescent readout of tubulin assembly performed in multi-well plates, where assembly is initiated by the addition of wild-type or mutant Tau. Figure 3 presents the results from simultaneous analyses of all mutants in assembly reactions. We observed that all mutants can promote assembly but with varying rates and m aximal assembly (Vmax) values. Phosphorylation of Tau does not promote assembly and we only observed three mutants that exhibited higher levels of tubulin assembly, mutations at 201, 181 and 169. The next stage in these analyses is to directly examine the effect of LRRK2 specific phosphorylation of tau on the assembly of tubulin. We therefore tested these mutants in in vitro kinase reactions with LRRK2 to determine which sites were preferred by LRRK2.

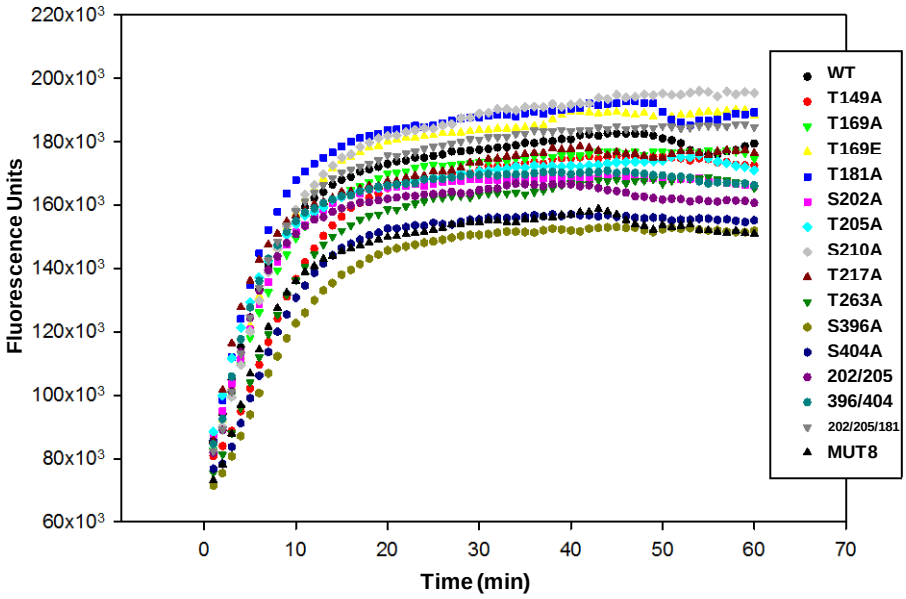

Figure 3. Tubulin assembly reactions with porcine tubulin were carried out with fluorescence detection as per manufacturer's protocol (Cytoskeleton Inc) on a B MG Omega plate reader. Assembly was initiated with addition of the indicated Tau proteins and fluorescence was read over 1hour.

Testing Tau mutants in LRRK2 kinase assays. We have generated mutants of Tau encompassing the LRRK2 sites mapped by mass spectrometry, as well as sites known to be important for tau regulation (i.e. 396/404 mutants). If we have mapped all LRRK2 mediated phosphorylation sites on Tau, we would expect some of these mutations to exhibit a lower level of phosphate incorporation in in vitro kinase assays. We tested the ability of LRRK2 to

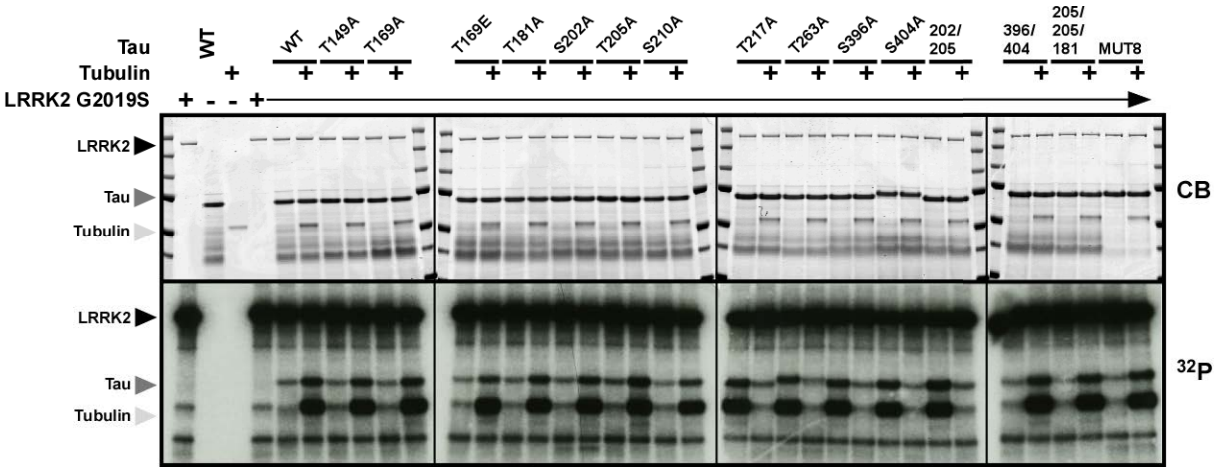

Figure 4. Phosphorylation analysis of Tau mutants. The indicated mutant recombinant tau was reacted with LRRK2 G2019S (970-End) protein in the presence or absence of tubulin with γ32P-ATP. Reaction products were resolved by SDS-PAGE and phosphorylation was

phosphorylate these recombinant proteins and visualized the reaction products by SDS-PAGE and autoradiography **FIGURE 2B**. Unfortunately, we observed little to no change in any of these sites when incubated with or without tubulin. We also observed similar modifications of the

MUT8 protein which harbors 8total phosphosite mutants. These data indicate that there are more LRRK2 phosphosites on Tau than we have uncovered and there is a future need to investigate the sites of LRRK2 modification.

Key Research Accomplishments

- LRRK2 can directly phosphorylate Tau and this phosphorylation is enhanced with splice variants of Tau
- LRRK2 Phosphorylation of Tau is enhanced in the presence of tubulin
- LRRK2 phosphorylates more than 6 sites on Tau in vitro

Conclusions

We have found that LRRK2 directly modifies the protein Tau on multiple sites. It was reported elsewhere that this was the case but that LRRK2 required tubulin in these in vitro reactions. Our results indicate that to be the case for only the longest splice form and that LRRK2 can modify shorter splice forms of tau directly. We mapped several LRRK2 phosphorylation sites on tau but more work is needed to ascribe function to these modifications and the entire complement of LRRK2 mediated modifications. If time and funds permitted we would have enjoyed the opportunity to elucidate the physiological role of these modifications as they may pertain to two of the most common neurodegenerative diseases in man.

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Appendices: NA

Supporting Data: Figures embedded, NA